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Corecruitment of the Grg4 repressor by PU.1 is critical for Pax5-mediated repression of B-cell-specific genes

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PU.1 and Pax5 are important regulators of immunoglobulin heavy-chain (IgH) gene expression in B lineage cells. We have previously shown that PU.1 can potentiate the transcription of an IgH HS1,2 enhancer-linked reporter gene, and that Pax5 represses the same enhancer in transient transfection assays. Here we report that PU.1, like Pax5, can recruit and physically interact with a member of the Groucho family of co-repressors, Grg4. As a consequence, PU.1 in conjunction with Pax5 represses enhancer function in a position-dependent manner when Grg4 is recruited. Interestingly, Grg4 levels decrease following B-cell activation, suggesting temporal regulation of Grg4. Moreover, the joining-chain promoter, with an activity pattern and architecture resembling HS1,2, can also be repressed by the combinatorial action of Pax5/PU.1/Grg4. These data indicate that Pax5 depends on PU.1, acting in *cis*, for stable recruitment of Grg co-repressors to B-cell-specific genes.

Keywords: PU.1; Pax5; Grg4; B-cell

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INTRODUCTION

A number of transcription factors have been defined as key components in haematopoietic development. The targeted deletions of the genes encoding two such transcription factors, the ets protein PU.1 and the paired box protein Pax5, have revealed them both to be required for the successful completion of B-cell development (Scott *et al*, 1994; Urbánek *et al*, 1994). Experimental evidence indicates a role for these transcription factors as coordinators of ordered genetic programmes involving activation of lineage-restricted genes concurrent with the active repression of alternative genetic pathways (reviewed in Schebesta *et al*, 2002). The immunoglobulin heavy-chain (IgH) HS1,2 enhancer is located at the far 3′ end of the IgH locus and displays a B-lymphoid-restricted activity (Arulampalam *et al*, 1997). Both PU.1 and Pax5 have been shown to regulate HS1,2 (Linderson *et al*, 2001), and Pax5 is a well-established repressor of this enhancer (Singh & Birshtein, 1996).

Previously, it has been shown that Pax5 can interact with a transcriptional co-repressor of the Gro/TLE (Groucho/transducin-like enhancer of split) family, Grg4 (Stifani *et al*, 1992; Eberhard *et al*, 2000). A puzzling aspect of Grg4-mediated Pax5 transcriptional repression is how target gene specificity is achieved, when Pax5 and Grg4 are present at a time when genes that actually require Pax5 for transcriptional activation, such as CD19, are in fact expressed. Explicitly, a molecular explanation of how Pax5-activated genes are positively regulated in an environment of negatively acting transcription factors has so far not been forthcoming. It has previously been shown in *Drosophila* that the transcriptional activator Dorsal can be converted from a transcriptional activator to a repressor by cooperating with another DNA-binding factor, which facilitates stable recruitment of Groucho to selected target genes (Valentine *et al*, 1998). This observation, in combination with the different functions ascribed to Pax5, suggested that the recruitment of Grg4 by Pax5 may not be obligatory but rather a consequence of cooperation between Pax5 and surrounding factor(s) when in the appropriate setting. Our previous observation that PU.1 is critical for Pax5-mediated repression of the IgH HS1,2 enhancer supports such a model (Linderson *et al*, 2001). Here we report that Grg4 can interact physically with PU.1 and that recruitment of Grg4 is critical for Pax5-dependent repression of an HS1,2-linked reporter gene.

RESULTS

Repression of HS1,2 is dependent on DNA architecture

The observation that Pax5-mediated inhibition of the enhancer required DNA binding of Pax5 (Neurath *et al*, 1995), as well as the binding of PU.1 to its cognate site (Linderson *et al*, 2001), suggested that the underlying molecular mechanism of repression would involve interaction between these two factors. To assess whether the spacing between the Pax5- and PU.1-binding sites of HS1,2 was functionally important for repression, we used the previously described –34SV–wt construct (Linderson *et al*, 2001)

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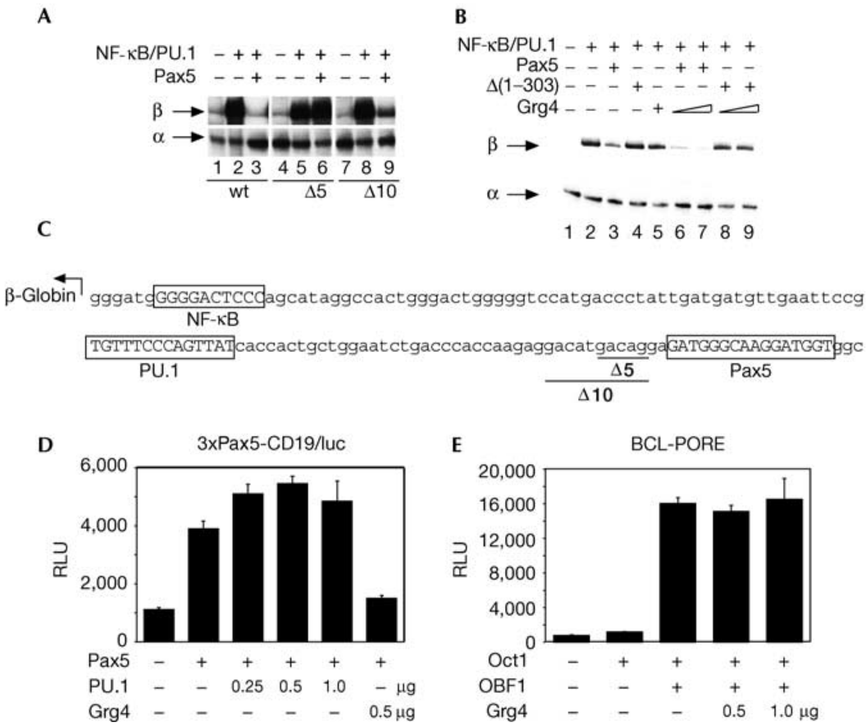

Fig 1 | Pax5-mediated repression of HS1,2-derived reporter constructs is dependent on position and PU.1. (**A**) RNase protection assay of RNA from COS-7 cells transfected with the pβ34SV–wt, –Δ5 or –Δ10 reporter constructs. Reporter gene activity (human β-globin, represented by β) was measured in transiently transfected COS-7 cells coexpressing p50/p65, PU.1 and Pax5 as indicated above each lane. The expression of human α-globin mRNA from the reference plasmid serves as a control for transfection efficiency (represented by α). (**B**) Pax5 lacking the octapeptide and C-terminal transactivation domain, Δ(1–303), cannot repress transcription. COS-7 cells were transfected with the pβ34SV–wt reporter and indicated transactivator plasmids before RNase protection assay of β-globin transcription. Two concentrations of Grg4 expression vector were used: 0.5 µg (lanes 6 and 8) and 1 µg (lanes 5, 7 and 9). (**C**) Promoter/enhancer sequence linked to the β-globin reporter gene. The Δ5 and Δ10 deletions are indicated. (**D**) Pax5-driven transcription is not repressed by titration of the pcDNA3-PU.1 vector, as indicated, into the system. The pKW2T–Grg4 is used as a control for repression. (**E**) Oct1-driven transcription is not repressed by Grg4. (**D**,**E**) Luciferase-derived light units are displayed in relation to β-galactosidase-derived relative light units (RLU). Experiments were performed in triplicate.

and two constructs in which deletions were introduced between the Pax5 and PU.1 sites, –34SV–Δ5 and –34SV–Δ10. Activation of reporter gene transcription from the three constructs by p50, p65 and PU.1 was comparable (Fig 1A, compare lanes 2, 5 and 8). The expression of Pax5 lowered the expression of the wt construct (compare lanes 2 and 3) but left the Δ5 construct virtually unaffected (compare lanes 5 and 6). Deletion of an entire helical turn (the Δ10 construct) largely restored Pax5-mediated repression (compare lanes 8 and 9). Thus, Pax5-mediated repression of the HS1.2 enhancer is context dependent.

The octapeptide and transactivation domain of Pax5 are interaction surfaces for the co-repressor Grg4 (Eberhard *et al*, 2000). To investigate the potential importance of these sequences in the Pax5-mediated repression of HS1,2 reporter gene activity, a deletion mutant of Pax5 lacking the octapeptide and the C-terminal activation domain (Δ(1–303); Fig 1B) was introduced into the test system. Again, Pax5 represses reporter gene transcription (Fig 1B, lane 3) whereas the deletion mutant does not (lane 4). The addition of exogenous Grg4 had little or no effect (lane 5) on β-globin expression. Pax5 in combination with Grg4 does however strongly suppress transcription (lanes 6 and 7), an effect that is eliminated when the Grg4 interaction surfaces are absent (lanes 8 and 9). It should be pointed out that Grg proteins have been shown to be expressed in a wide variety of tissues and cell lines (Stifani *et al*, 1992; Brantjes *et al*, 2001), and it is therefore possible that repression of our reporter gene construct by Pax5 is aided by endogenous Grg proteins and is strengthened by additional Grg4 expression. Our data are not compatible with previous data, which proposed that Pax5 could sequester PU.1 and compete for coactivator binding (Maitra & Atchison, 2000). To test for a possible sequestration mechanism, we used a minimal promoter construct containing three Pax5-binding sites driving a luciferase reporter gene. In contrast to the data by Maitra & Atchison (2000), Pax5-driven transcription (Fig 1D, lane 2) was not affected by increasing PU.1 expression in this system (lanes 3–5), whereas Grg4 efficiently repressed Pax5-driven transcription as previously shown (lane 6). To ensure that Grg4 does not have a general effect on luciferase transcription, we performed a control experiment with a reporter gene containing a single binding site for Oct factors in its promoter (BCL-PORE). Figure 1E shows that transcription activated by Oct1 is not affected by the coexpression of Grg4, showing that Grg4-mediated repression does not occur in an unspecific way.

Grg4 interacts with PU.1 both physically and functionally

In the search for a potential Pax5/PU.1 cofactor, Grg4 was an obvious candidate given its capacity to interact with Pax5. Grg4 has furthermore been shown to interact with other important lymphocyte-restricted factors, such as BLIMP-1 and the p65 subunit of NF-κB (Ren *et al*, 1999; Tetsuka *et al*, 2000). This implies that the data presented in Fig 1A may also depend on the presence of p65 at the template. However, we chose to focus our attention on PU.1 because we had previously noted a role for PU.1 in this context (Linderson *et al*, 2001). Bacterially expressed GST-Grg4 protein was therefore used as bait in GST pull-down experiments and, as shown in Fig 2A, both PU.1 and Pax5 can interact in solution with Grg4. In contrast, no interaction between the ets protein Elf-1 and Grg4 was observed (Fig 2A). In a control experiment, we further confirmed that p65 could interact with GST-Grg4 in solution whereas the other NF-κB subunits c-Rel, p50 and p52 could not (Fig 2A). In a subsequent co-immunoprecipitation (Co-IP) assay, we tested whether the interaction between PU.1 and Grg4 also occurred *in vivo*. Indeed, Grg/TLE protein was found in a complex with PU.1 (Fig 2B, lane 1). No Grg/TLE protein was visible in the sepharose A control (lane 2) or in precipitates with extracts where only Grg4 had been over-expressed (lane 3).

We next investigated whether PU.1-driven transcription could be a target for Grg4-mediated repression. Transcription driven by multiple PU.1-binding sites could be repressed by Grg4 in a dose-dependent manner (Fig 2C, compare lane 2 to lanes 3 and 4). This is in line with the previously reported Grg4-mediated repression of a minimal promoter containing multiple Pax5-binding sites (Eberhard *et al*, 2000).

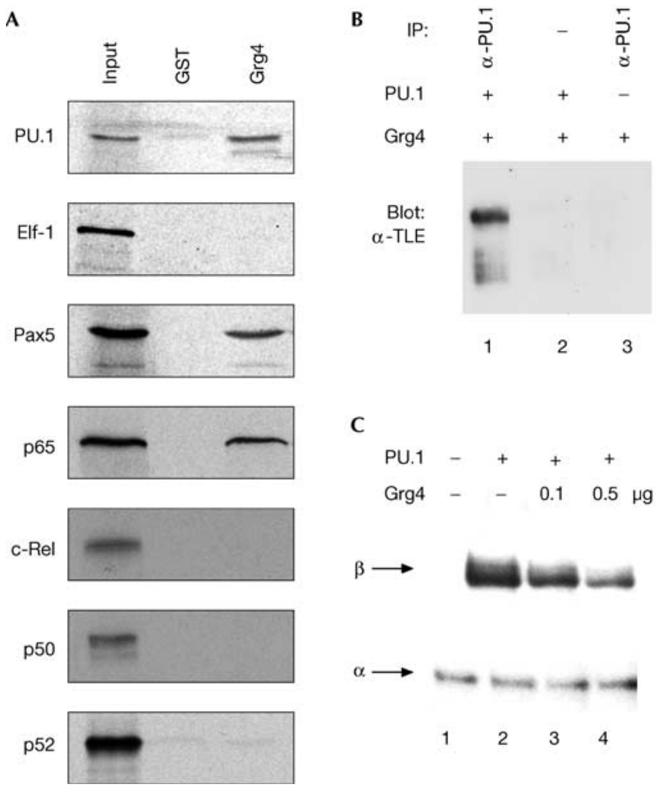

Fig 2 | Grg4 can interact with PU.1 both physically and functionally. (**A**) *In vitro* complex formation of Grg4 with PU.1. GST pull-down experiments where murine Grg4 fused to GST, or GST serving as a negative control, have been used as bait for the ^{35}S-labelled interaction candidates indicated to the left in the figure. The first lanes (Input) contain 10% of the input of the *in vitro*-translated candidate proteins. The input levels of GST and GST-Grg4 are shown as a supplementary figure online. (**B**) *In vivo* complex formation of PU.1 and Grg4. Western blot displaying the TLE content complexes with PU.1 in nuclear extracts from 293A cells. The cells had been transfected as indicated above the blot. An α-PU.1 antibody was used for immunoprecipitation, and a monoclonal rat pan-TLE antibody for western blot. (**C**) Grg4 interacts functionally with PU.1. RNase protection assay of the human β-globin transcript (represented by β) in 293A cells derived from the pβ34SV3–4xPU.1 plasmid when activated by the co-transfection of pcDNA3–HA–PU.1 (0.25 μg). The amount of pKW2T–Grg4 expression vector was titrated as indicated above the radiogram. α-signal as in Fig 1A.

Grg4 is corecruited to HS1,2 by PU.1 and Pax5

The hypothesized recruitment of Grg4 to an HS1,2 template was investigated next by use of the –34SV–wt construct. To avoid the possible involvement of p65 in our test system, we chose to use

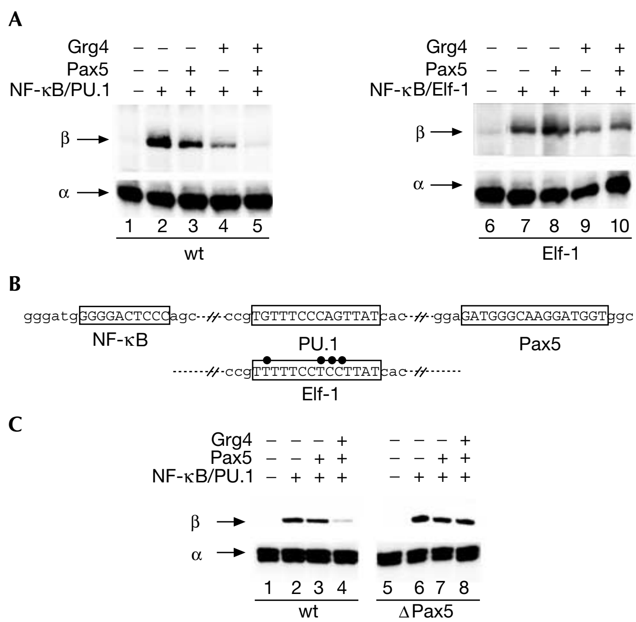

Fig 3 | Grg4 can repress transcription driven by an IgH HS1,2-derived element in a PU.1- and Pax5-dependent manner. (**A**) RNase protection assay showing the β-globin signal (β) in COS-7 cells derived from the pβ34SV–wt construct when regulated by the overexpression of p50/c-Rel, PU.1, Pax5 and Grg4 as indicated above each lane. (**B**) Reporter gene activity (β) derived from the pβ34SV–Elf-1 construct in COS-7 cells when activated by the coexpression of p50/c-Rel and Elf-1 in combination with Pax5 and Grg4 as shown above the radiogram. α reference as in Fig 1A. The point mutations generating an Elf-1-binding site in the HS1,2 fragment are depicted below the wt sequence. The Elf-1-binding sequence has been described previously (Thompson *et al*, 1992). (**C**) β-Globin expression is repressed by Grg4 in the presence (wt, lane 4) but not in the absence (ΔPax5, lane 8) of the Pax5-binding site (boxed sequence).

p50 and c-Rel in combination with PU.1 for transactivation. While Pax5 and Grg4 reduce reporter gene activity to some extent on their own (Fig 3A, compare lanes 2–4), a robust inhibition of reporter gene activity is again observed (fivefold) when Pax5 and Grg4 are added in combination (lane 5). This observation was further supported using a reporter gene construct in which the PU.1-binding site had been replaced with a binding site for Elf-1 (Linderson *et al*, 2001). Using the same activation conditions as in Fig 3A, elevation of the reporter gene signal was observed (Fig 3B, lane 7). However, addition of Pax5 or Grg4 or in combination does not appear to repress reporter gene activity (compare lane 7 to lanes 8–10). Pax5-mediated repression of the HS1,2 enhancer consequently requires the presence of PU.1 for the recruitment of Grg4. Because Pax5 is able to interact directly with the co-repressor, we had hypothesized that Grg4 would be corecruited by Pax5 and PU.1. The previous report, claiming that Pax5 repressed PU.1-dependent transcription in the absence of its DNA-binding motif, prompted us to test this directly, simply by deleting the Pax5-binding site of HS1,2 (shown in Figs 1,3). Figure 3C shows that an intact Pax5-binding site is essential, and a prerequisite for Grg4-mediated repression (compare lanes 4 and 8).

Grg4 can exert repression on the joining-chain promoter

Our findings raised the question whether additional B-cell-specific *cis* elements would be subject to a similar type of coregulation. The expression of the joining (J) chain coincides with late B-cell development (Lamson & Koshland, 1984). The J-chain promoter is an additional B-cell-specific *cis* element that binds Pax5 and PU.1, and the two sites are separated by approximately five helical turns of DNA. The J-chain promoter has also been established as a direct genetic target for Pax5 whose expression negatively correlates with J-chain expression (Mikkola *et al*, 2002).

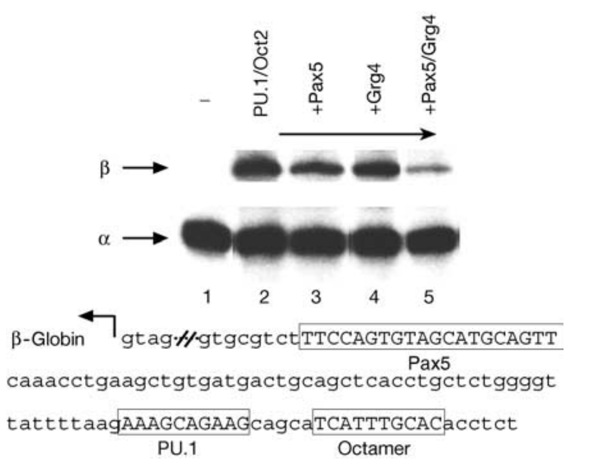

Fig 4 | Grg4 can inhibit a J-chain promoter-dependent reporter gene. The −34SV–Jp was transfected into 293A cells with or without expression vectors for PU.1, Oct2, Pax5 and Grg4 as indicated. The transcription of the reporter gene (β, as in Fig 1A) was analysed by ribonuclease protection assay. α reference as in Fig 1A. The relevant part of the cloned J-chain promoter sequence is shown below. Note that approximately 140 bp of the sequence upstream of the Pax5-binding site have been omitted in the figure, but can be found in the original publication (Sigvardsson *et al*, 1993).

The analysis of a J-chain promoter-driven reporter gene (Fig 4) further supported our idea about cooperative recruitment of Grg4 by Pax5 and PU.1. Pax5 and Grg4 had little effect on reporter gene activity on their own (lanes 3 and 4), whereas a combination of the two gave a substantial repression of transcription (lane 5).

Decreased levels of Grg proteins in activated B cells

Both J-chain promoter and HS1,2 enhancer activity increase after B-cell activation, in part due to decreased Pax5 levels. As our findings suggested a role for Grg4 in the regulation of these *cis* elements, the level of Grg proteins during the course of B-cell activation was determined. As can be seen in Fig 5, a relative decrease in the nuclear Grg content (lanes 2 and 3 compared with lane 1) is observed in $B220^+$ B cells. To ensure that the decrease in nuclear TLE proteins is not merely due to general degradation of protein in the nuclear extracts, a duplicate gel was analysed for its content of Oct2. The result is displayed in the lower panel of Fig 5.

DISCUSSION

Tissue-specific gene expression is often brought about by transcription factors with specific, or restricted, tissue distribution, acting in cooperation with more generally expressed cofactors. We have shown that Pax5 and PU.1 cooperatively can recruit the co-repressor Grg4 to the HS1,2 enhancer and thereby acquire repressor function. The recruitment of the cofactor is dependent on the architecture of the regulatory element (Fig 1A), and exemplifies how transcription factors can be involved in the activation of transcription of some target genes at the same time as in the repression of others. The J-chain promoter is another well-known target for Pax5-mediated repression that is additionally regulated by PU.1, and we find that this promoter can be targeted by Grg4. Notably, the J-chain promoter is not regulated by NF-κB proteins. The loss of Grg4-induced repression as a consequence of the PU.1/Elf-1-binding site substitution (Fig 3B) further supports the idea that PU.1 is a cooperative partner of Pax5 in the recruitment of Grg factors.

Pax5 and PU.1 cannot independently recruit Grg4 (as opposed to obligate Groucho-binding repressors such as Hairy and Engrailed). Hence, these transcription factors appear to be positioned on DNA in a precise configuration in relation to one

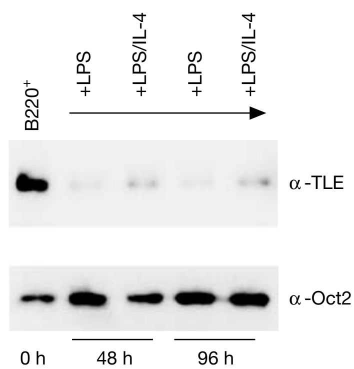

Fig 5 | Nuclear Grg protein levels decrease following late B-cell differentiation. Nuclear extracts were prepared from MACS-purified splenocytes. In all, 5 μg protein of each sample was analysed by western blot with a pan-TLE antibody (upper panel) or an α-Oct2 antibody (lower panel).

another to form a platform for Grg4 recruitment. PU.1 and Pax5 have further been suggested to downmodulate transcription by means of the κ light-chain 3′ enhancer (Shaffer *et al*, 1997; Liu *et al*, 1999; Maitra & Atchison, 2000). The two sites are spaced by approximately one helical DNA turn, and could theoretically form an interaction surface for Grg factors when bound by PU.1 and Pax5. Roque *et al* (1996) have further reported that these DNA motifs can be occupied simultaneously during earlier stages of B-cell development. It could be speculated that the co-repressor recruitment may be used in the regulation of a whole set of genes with B-cell-restricted expression. The decrease of nuclear Grg factors that we observed (Fig 5) upon B-cell activation would hence result in the simultaneous relief of repression of this collection of genes and aid terminal differentiation. Our observation gains further support in a recent report in which gene expression profiles in B cells and plasma cells were compared and it was found that Grg4 transcript levels were reduced in the plasma cell population (Underhill *et al*, 2003). We have previously reported that activation of HS1,2-driven transcription precedes the downregulation of Pax5 expression (Andersson *et al*, 1996). This observation was puzzling at the time, but correlates with a change in cofactor accessibility. The three gene regulatory elements discussed above, the HS1,2 enhancer, the J-chain promoter and the κ light-chain 3′ enhancer, are all involved in gene regulatory processes that ultimately control the secretion of immunoglobulins. The production of antigen-specific antibodies fulfils a central role of B-cell existence, and it seems rational that genes encoding components of antibodies, or factors that are important for their secretion, are expressed in a coordinated fashion.

MATERIAL AND METHODS

Plasmids and cloning. The pβ34SV3–wt and pβ34SV3–Elf-1 have been described previously (Linderson *et al*, 2001). The pβ34SV3–Δ5, pβ34SV3–Δ10, pβ34SV3–ΔPax5, pβ34SV3–Jp and pβ34SV3–4xPU.1 constructs were generated using standard mutagenesis and cloning procedures, and details are available on request. The BCL-PORE vector was a kind gift from H. Schöler. pEVRF2–HA–Elf-1 was provided by A. Nordheim. pcDNA3–PU.1 was provided by H. Singh. 3xPax5-CD19/luc (Czerny & Busslinger, 1995) and pKW2T–Grg4 (Eberhard *et al*, 2000) have been described previously. pKW10–hBSAPΔOCT was constructed by T. Czerny and pKW2ThBSAPΔOCT(1–303) by D. Eberhard. Grg4 cDNA was cloned into pSCT1 (S Rusconi) to generate pSCT1-Grg4.

Cell lines and transfections. Cos-7 and 293A cells were transfected using a standard calcium phosphate co-precipitation protocol or FuGENE6 (Roche Diagnostics). For ribonuclease protection analysis, 10 μg β-globin reporter gene and 4 μg reference plasmid (α2-globin) were transfected. The assays were carried out 36 h after transfection according to standard procedure. Typical expression vector amounts were as follows: 1 μg of Rc/CMV-p50 and Rc/CMV-cRel, 2 μg of pcDNA3–HA–PU.1 and pEVRF2–HA–Elf-1, and 0.5 μg of pKW10–hBSAP and pKW2T–Grg4 per transfection (each transfection supplemented with empty vector to give 20 μg DNA in total). Luciferase reporters were transfected into 293A cells: 2 μg of 3xPax5-CD19/luc or 1 μg of BCL-PORE/luc with 0.5 μg of appropriate expression vectors and 10 ng of a β-galactosidase reference vector. Luminescence was measured with the Dual-Light® System (Applied Biosystems). For Co-IPs, 2 μg expression vector, 1 μg pβ34SV3–4xPU.1 and 0.1 μg T-antigen-expressing vector (5.1 μg DNA/transfection) were transfected into 293A cells. Phorbol 12-myristate 13-acetate (PMA; 50 ng/ml) was added 20 h before harvest for increased protein expression.

Co-immunoprecipitation, western blot and GST pull-downs. For Co-IPs, nuclear extracts were precleared with Protein A Sepharose™ CL-4B (40 μl, 50%, Amersham, Pharmacia) in IP buffer (20 mM HEPES pH 7.4, 0.1 M NaCl, 5 mM MgCl2, 0.2 mM EDTA, 2% glycerol, 1% NP-40, 1 mM PMSF). Co-IPs were performed with 40 μl (50%) beads containing 50 μg/ml α-PU.1 (sc-352, Santa Cruz). Samples were analysed by western blot. A pan-TLE antibody (S. Stifani) or α-Oct2 (sc-233, Santa Cruz) was used for detection with the ECL™ system (Amersham, Pharmacia). GST pull-downs were carried out as described (Eberhard *et al*, 2000).

Primary B cells. B cells were purified from mouse spleens using positive selection on B220^{+} magnetic beads as described by the manufacturer (SuperMacs, Miltenyi Biotech). The B cells were cultured in RPMI supplemented with 10% FBS, 20 U/ml penicillin and 20 μg/ml streptomycin, and were activated with LPS (20 μg/ml) alone or in combination with IL-4 (10 ng/ml) for 48 or 96 h.

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